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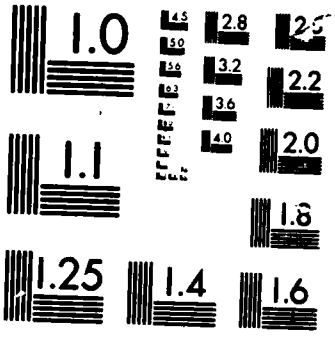

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A rat anti-murine gamma interferon (MuIFNγ) neutralizing monoclonal antibody (MAb) was used to investigate the effect of MuIFNγ on the development of antiviral resistance, Fc receptor expression, and Ia antigen in macrophages. These studies were carried out in cultures of C3H/HeJ peritoneal macrophage cultures in order to determine: 1) whether MuIFNγ can influence the expression of the above parameters, 2) the relative quantity of MuIFNγ required to mediate each effect, and 3) the amount of anti-MuIFNγ MAb required to neutralize each MuIFNγ-mediated effect. The results of these studies established that macrophages are at least as sensitive to the antiviral action of MuIFNγ as other cell types tested, however,

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19. Abstract cont.

Substantially more MAb was needed to neutralize MuIFNγ-mediated antiviral activity in macrophages. It was also found that 10-15 times more MAb was required to inhibit MuIFNγ-induced Fc receptor expression than was required for Ia antigen expression. These latter findings confirm and extend previous observations, which indicate the MuIFNγ induction of enhanced expression of these two macrophage membrane differentiation markers occurs through distinct mechanisms.

During the course of a sublethal Listeria monocytogenes infection, all three IFN classes (α, β, γ) are produced by the host. In addition to their antiviral activities, IFNs can mediate a variety of different effects which could conceivably have a bearing on the expression of host nonspecific and/or specific resistance mechanisms. Interferons have been reported to suppress the intracellular multiplication of a variety of nonviral intracellular organisms. Since Listeria is a facultative intracellular bacterium, capable of multiplying in both professional and nonprofessional phagocytic cells of infected organs, a model of intracellular Listeria infection of nonprofessional phagocytes (fibroblasts) was developed. It was found that IFN α/β and IFNγ both inhibit intracellular Listeria proliferation. Moreover, during intracellular Listeria proliferation, infected fibroblasts produce IFN α/β. Thus, in vivo, Listeria-infected nonphagocytic cells, such as hepatocytes, may be an important source of IFN α/β which through its inhibitory action on intracellular Listeria multiplication, may serve to restrict the magnitude of infection faced by the host.

FINAL REPORT
N00014-83-C-0407
Actions of Interferons on Macrophages

I. MuIFNγ-Mediated Effects on Macrophages

Previously, we reported on the generation of a rat:mouse hybridoma (R4-6A2) which secretes rat anti-MuIFNγ neutralizing monoclonal antibody (MAb). This MAb was used to establish that MuIFNγ was the factor present in crude lymphokine preparations which was responsible for priming macrophages for enhanced tumoricidal activity (1). Since this finding, studies have been undertaken to both produce and purify sufficient quantities of the MAb to further study MuIFNγ-mediated effects (2).

The ability of the R4-6A2 anti-MuIFNγ MAb to neutralize several different acivities of MuIFNγ was recently examined (3). The MAb was found to neutralize the ability of MuIFNγ to inhibit the growth (anti-proliferative activity) of fibroblast cells in culture. The ability of MuIFNγ to render cells of a heterologous species (rat) resistant to viral replication was also neutralized by this MAb. The MAb neutralizing titers for MuIFNγ antiviral activities, on both homologous (murine L929B fibroblast) and heterologous (rat fibroblast) cells, were inversely proportional to the antiviral activity titers on each cell type (2). This linear relationship between the MAb neutralizing titer and the quantity of MuIFNγ (antiviral activity) neutralized was not observed in the earlier studies on MAb neutralization of MuIFNγ-induced macrophage tumoricidal activity (1). However, the quantity of MAb required to eliminate the macrophage activating potential of MuIFNγ, as assessed by the enhanced ability of these cells to destroy tumor targets, was in excess of 20-100 times that required to abolish antiviral activity on fibroblasts.

Macrophages are thought to be very sensitive to MuIFNγ-mediated effects. One possible explanation as to why more MAb is required to neutralize MuIFNγ-induced effects in macrophages, is that macrophages may be relatively more sensitive to MuIFNγ than fibroblasts. Therefore, more MAb would be needed to achieve a higher degree of neutralization of MuIFN -mediated effects in macrophages,than is required in less sensitive cell types. Studies were undertaken in collaboration with Dr. Stephanie Vogel (Uniformed Services University, Bethesda, MD) to examine the quantity of MuIFNγ required to evoke antiviral resistance, as well as the enhanced expression of certain macrophage membrane markers. Thus, based on the quantity of MuIFNγ needed to elicit macrophage resistance to virus infection, it would be possible to relate the degree of MAb neutralization achieved to the quantity of antigen (MuIFNγ antiviral activity) required to protect cells of varying sensitivities to virus infection, and to other MuIFNγ-mediated effects in macrophages.

In order to analyze MuIFNγ antiviral activity in macrophages, cultures of peritoneal macrophages were established from C3H/HeJ mice. These cells proved permissive for vesicular stomatitis virus (VSV) replication and were used to study the antiviral action of MuIFNγ by means of both viral cytopathic and virus yield assays. Based on relative antiviral activity in fibroblast cultures, it was observed that as little as 0.5 MuIFNγ laboratory reference units induced a state of antiviral resistance in macrophages, and that these phagocytic cells exhibited approximately the same sensitivity to the antiviral activity of MuIFNγ against VSV challenge as fibroblasts. Moreover, the quantity of MAb required to neutralize the antiviral activity of MuIFNγ half maximally, was 20-100 times greater than that required for MuIFNγ antiviral activity on fibroblasts (3). This finding was similar to that observed for MuIFNγ-induced macrophage activation (1). These results suggest that since macrophages and fibroblasts exhibit similar sensitivities to MuIFNγ-induced antiviral resistance, other reasons must account for why considerably more neutralizing MAb is needed to neutralize MuIFNγ-mediated effects in macrophages, than is required in cultures of fibroblasts.

MuIFNγ-induced enhanced expression of macrophage FcR and Ia antigen expression was also examined in cultures of C3H/HEJ macrophages (3). It was found that both recombinant or natural MuIFNγ preparations elicited increased FcR and Ia antigen expression over approximately the same concentration ranges and periods of time. As with MuIFNγ-induced antiviral resistance in both macrophages and fibroblasts, 5.0 units of MuIFN resulted in maximum FcR and Ia expression. The capacity of the R4-6A2 anti-MuIFNγ MAb to neutralize the ability of MuIFNγ to induce these macrophage differentiation markers was assessed. To be consistent with the definition of the neutralization titer for antiviral activity, the highest dilution of MAb that reduced the FcR- or Ia-inducing effects of 5.0 units of MuIFNγ by 50% was chosen as the endpoint in the neutralization assay. The neutralization titer of the MAb to reduce MuIFN induced FcR half maximally was 10^3. In contrast, the neutralizing titer of the MAb for MuIFNγ-induced Ia expression was $1\text{-}5\times10^4$. Thus, significantly less MAb was required to inhibit MuIFNγ-induced Ia expression than was required for either MuIFNγ-induced antiviral resistance or FcR expression.

II. Antimicrobial Functions of Interferons.

The response of mice to the bacterium, Listeria monocytogenes, offers an excellent model for studying the possible roles of interferons (IFNs) in preimmunity resistance and acquired specific resistance to a facultative intracellular pathogen. Results of studies carried out in this laboratory have established that during a sublethal immunizing Listeria infection all three classes of IFN are produced. Moreover, the infected host acquires an augmented capacity to produce all three IFNs in response to appropriate IFN-inducing agents (4). In view of these findings, and the reported activities of IFNs which could influence the course of a bacteria infection; such as (a) suppressing proliferation of intracellular bacteria, (b)

enhancing the bactericidal activities of macrophages, and (c) possible immunomodulatory activities, it is conceivable that IFNs produced during listeriosis may function in resistance. A goal of our research is to determine the functions of each IFN in resistance to a nonviral infectious disease, using murine listeriosis as an experimental model.

The following studies have either been performed, or are currently underway to establish whether an IFN functions in resistance to Listeria, and if so, its role in either specific and/or nonspecific resistance.

Examination of a possible role of IFNs in nonspecific resistance to Listeria. In vitro, IFNs have been found to suppress the intracellular proliferation of certain bacteria. Histological examination of Listeria-infected livers has established that this bacterium proliferates in both professional and nonprofessional phagocytic cells. Therefore, if IFNs inhibit intracellular Listeria multiplication in vivo, such an action would constitute an important aspect in nonspecific resistance to this pathogen. An in vitro model of Listeria infection of nonprofessional phagocytes was developed during the past year inorder to investigate possible effects of each IFN class on intracellular multiplication of this gram-positive facultative bacterium. This model uses murine embryo fibroblasts and the aminoglycoside antibiotic, gentamicin sulfate, at a concentration that is listericidal for extracellular, but not intracellular bacteria. The following is a list of our findings:

1. During exponential growth, the extracellular doubling time of Listeria is 25 minutes, whereas the intracellular doubling time is estimated to be 2.5 hr (Fig. 1). Quantitation of cell-associated Listeria is performed by lysing fibroblasts with 0.05% deoxycholate and then plating the lysate on agar.

2. During the intracellular proliferation of Listeria, infected fibroblasts produce substantial quantities of IFN (Fig 2). This Listeria-induced IFN has been characterized as to certain physicochemical properties and antigenicity (Table 1), and has been found to be indistinguishable from virus-induced IFN-β (5). Thus, one potential source of serum IFN-β detected during the peak of Listeria growth in the liver and spleen may be infected nonprofessional phagocytes, such as parenchymal cells.

3. Microscopic examination of Listeria-infected fibroblast cultures reveals that, following the intital infection procedure, approximately 1% of the fibroblasts are associated with Listeria. Internalized bacteria proliferate and eventually occupy the entire cytoplasm, after which the host cell is destroyed and bacteria are released into the culture medium. Cells that are both contiguous and distant to the infectious focus become infected. Subsequent cycles of this infectious process results in destruction of the fibroblast monolayer in two days.

4. The use of semi-solid agar overlays, to prevent the random dispersal of extracellular Listeria from destroyed cells, has allowed the microscopic observation of cell to cell spread of infection. The agar overlay permits only the radial spread of infection. The following are our observations on cell to cell spread of intracellular Listeria:

a) All cells adjacent to an infected fibroblast become infected.

b) Spread of Listeria is not contingent on the disruption of the infected cell. This can be suggested because even towards the center of an advanced focus of infection, many heavily infected cells possess intact plasma membranes.

c) In more recently infected cells, towards the periphery of the infectious focus, Listeria appears to be within phagocytic vacuoles. This would suggest that intercellular spread of Listeria may occur through phagocytosis.

d) In contrast to the total destruction of the infected monolayer maintained in fluid medium for 48 hrs, the infected cultures overlaid with agar for 48 hrs possess the same number of infectious foci as observed 24 hrs earlier, but each focus was larger and contained more dead cells. The progressive nature of Listeria infection of fibroblasts maintained under agar eventually became macroscopically evident as discreet plaques in the monolayer.

5. IFN$\alpha/\beta$and IFNγ suppress intracellular Listeria infection of fibroblasts. However, IFNγ appears to differ from IFNα/βin its anti-Listeria effect. This conclusion is based on results obtained with the Listeria plaquing technique mentioned above to assess the effect of IFNs on intracellular Listeria infection. Based on what has been reported for IFN-mediated effects that could conceivably inhibit intracellular multiplication of parasites, there are four potential sites where IFNs could affect inhibitory actions. These are: 1) initial association of Listeria with the cytoplasmic membrane; 2) the internalization process; 3) intracellular multiplication; and 4) the subsequent infection of neighboring fibroblasts. It has been found that if fibroblasts are treated with MuIFNγ prior to infection with Listeria, the resulting plaque number is greatly reduced. This MuIFNγ -mediated effect is dose dependent and requires 12-18 hrs to develop. Similar treatment of fibroblasts with MuIFNα/β has little, or no effect on bacterial plaque numbers. However, plaque size is reduced in the MuIFNα/β treated fibroblasts. These different inhibitory effects of the IFNs would suggest that MuIFNγ inhibits an early event in the infectious process, whereas MuIFNα/β may induce a cellular event which inhibits intracellular Listeria multiplication. If indeed, these MuIFNs differ in their inhibitory effects on the intracellular Listeria infectious process, then their combined actions might very well result in a synergistic anti-Listeria effect.

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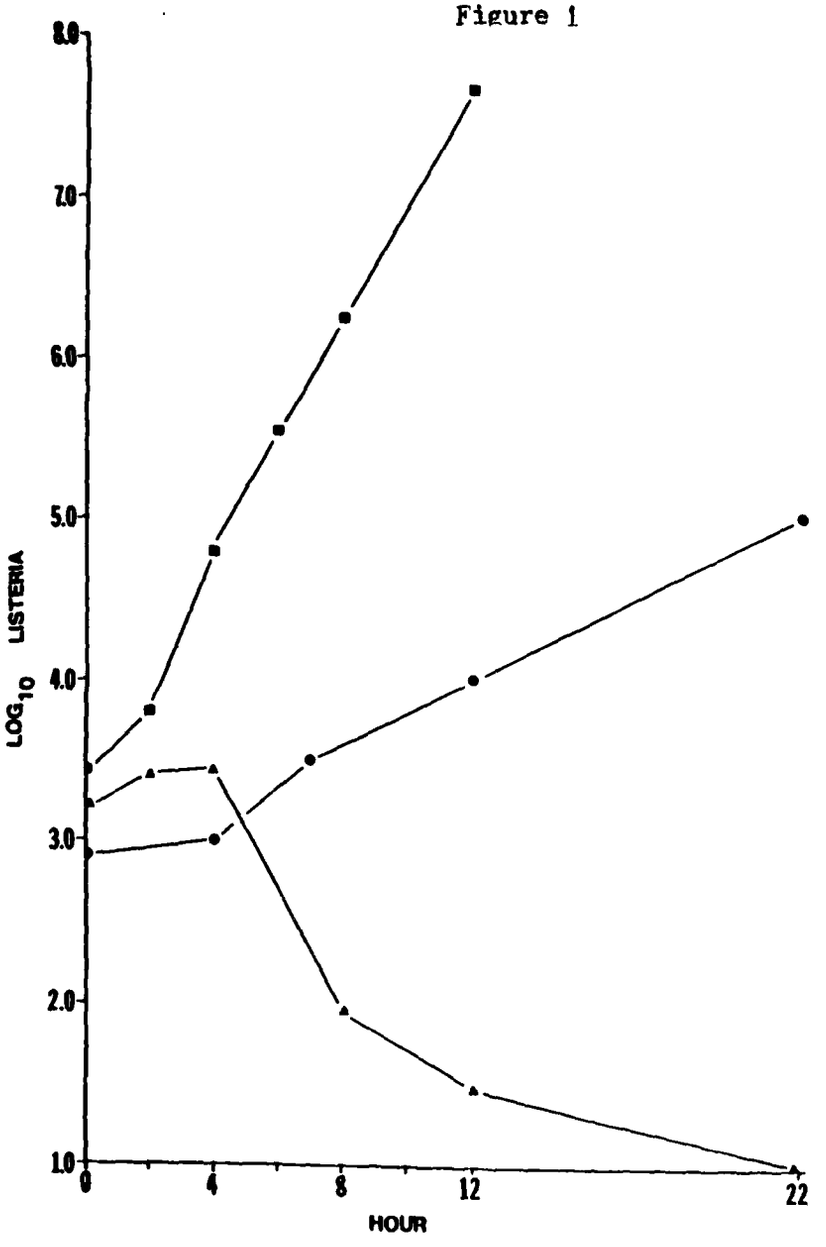
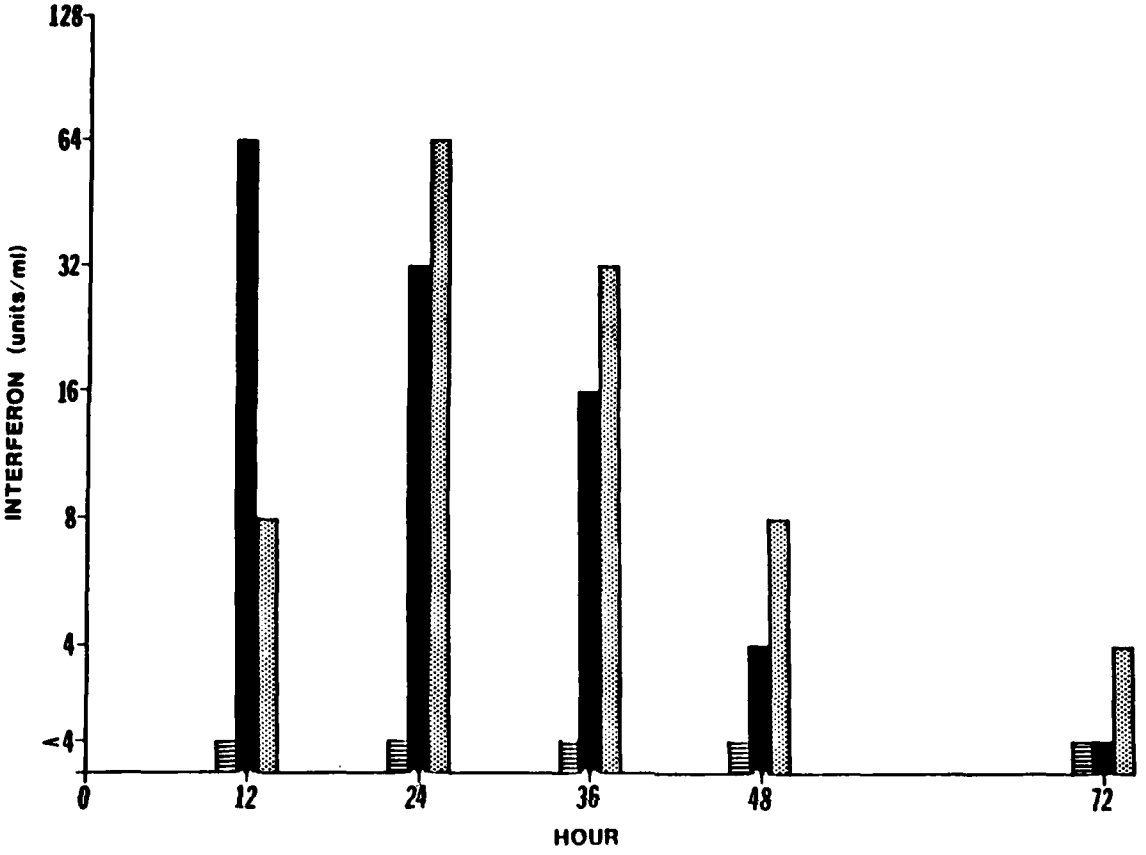
Figure 1



Intracellular and extracellular multiplication of Listeria monocytogenes. Listeria growth in EMEM containing no antibiotics (■); EMEM containing gentamicin sulfate (0.25 µg/ml) (▲); or in murine embryo fibroblast monolayers incubated with EMEM containing 0.25 µg/ml of gentamicin sulfate (●).

Figure 2



Listeria monocytogenes-induced IFN synthesis in murine embryo fibroblasts. Interferon titers (units/ml) in murine embryo fibroblast culture media at various intervals follow-the initial 2 hr Listeria adsorption period with ratios of infection of 10 Listeria/cell (solid bars); 0.5 Listeria/ cell (dotted bars); or no Listeria (striped bars).

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